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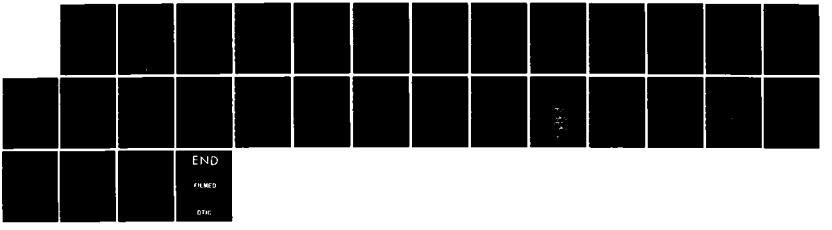

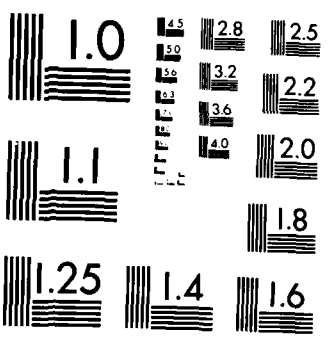

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A Microprocessor-Controlled, Multichannel Fluorimeter for Analysis of Sea Water

by

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2 Tables
6 Figures

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SUMMARY

The design of a multichannel fluorometer which provides good sensitivity and rapid data acquisition is described. The advantages of multidimensional fluorescence detection are discussed with special reference to the continuous monitoring of in vivo chlorophyll fluorescence in the marine environment. Preliminary experiments regarding chlorophyll determinations at a detection limit of 5×10^{-12} M along with detection linearity (r=0.9994) are used to evaluate the instrument design.

INTRODUCTION

Fluorescence spectroscopic techniques are characterized by low detection limits and selectivity. The detection limit of fluorescence is typically three to four orders of magnitude below that of absorbance. This advantage arises from the direct detection of photons using fluorescence in contrast to the measurement of a small difference in two large photon signals in absorbance.

The many parameters of fluorescence spectroscopy which can be exploited provide the advantage of excellent selectivity. Emission intensity is dependent on both the wavelengths of excitation and emission. Many fluorophores can be spectrally separated based on these two parameters alone. However, by incorporating other parameters such as fluorescence lifetimes, phosphorescence spectra and lifetimes, and polarization, even greater selectivity can be obtained. Many recent studies have cited the multiparameter advantage of luminescence spectroscopy and have utilized it in the analysis of multicomponent samples [1,2].

Advances in instrument design have been crucial to the development of methods which capitalize on the sensitivity and selectivity of fluorescence. The early instruments used simple bandpass filters to restrict the wavelengths of excitation and emission. Later, more sophisticated

spectrofluorometers incorporated two scanning monochromators for excitation and emission resolution. Both of these instrument types are still well represented in current literature. The most recent advances in instrumentation have been facilitated by improvements in computer technology and optoelectronic detection devices [3]. Improved computer technology allows the acquisition and subsequent analysis of large data sets while reducing operator time. The development of imaging devices such as vidicons, charge-coupled devices, and linear photodiode arrays has provided simultaneous multiwavelength detection. The linear photodiode arrays and intensified arrays are the newest of the multichannel detectors and offer several advantages over the older vidicons. These arrays are usually constructed as one-dimensional detectors in contrast to the two-dimensional vidicons. However, their good sensitivity, minimal lag, and minimal blooming provide superior performance for many applications [4,5].

Lorenzen [6] demonstrated that fluorescence is a sensitive method for the measurement of <u>in vivo</u> chlorophyll a in the ocean. He was able to detect as little as 10^{-11}M chlorophyll a by using a simple Turner model III fluorometer equipped with a red sensitive photomultiplier tube (PMT). This approach to chlorophyll monitoring has become increasingly popular [7,8] because of capabilities for

continuous data acquisition. The previous methods involved discrete sampling, filtering, extraction, and subsequent analysis by either the trichromatic method of light absorbance [9] or fluorescence [10]. These techniques were much more time consuming and offered less topographical information.

This paper presents the design of a portable, multichannel fluorometer (PMF) for use on board ship for analysis of fluorescent species in the ocean. Preliminary laboratory evaluation and experimental results will be discussed with regard to applications in the marine environment. The potential for shipboard operation in the continuous monitoring of *in vivo* chlorophyll a excitation and emission spectra will be described along with the apparent detection limits.

MATERIALS AND METHODS

Samples

Standard chlorophyll a was obtained from Sigma Chemical Company and the *Chlamydomonas reinhardi* from Carolina Biological Supply. The extracted chlorophyll a samples were collected on the November, 1982 cruise of the R.V. Gyre in the Gulf of Mexico*. Sample collection required filtering

* This cruise was in collaboration with Dr. D. R. Schink, Texas A&M University, College Station, Texas.

approximately 500ml of seawater through a Whatman GF/C filter. The filters were frozen for transport and storage. A tissue grinder facilitated extraction of chlorophyll a into 90% acetone, as described by Yentsch et al. [10].

Instrumentation

There were six characteristics which necessarily had to be incorporated into the design of the PMF: 1) portability 2) low detection limits 3) rapid data acquisition 4) multidimensional detection 5) automation capabilities 6) rugged construction. Portability is required because many fluorescent species, especially in biological systems, must be investigated in their natural environment. Therefore, the instrument must be easily transportable and capable of operation in a nonlaboratory environment. A low detection limit is crucial because concentrations of chlorophyll a in the ocean are typically 10^{-8}-10^{-11} M. The characteristics of rapid data acquisition and multidimensional detection are needed to provide the spectral information with sufficient time resolution to allow topographical mapping. It is also desirable to incorporate capabilities for automation into the design of the instrument because it will often be operating continuously. Finally, the construction of the fluorometer must be sufficiently rugged to withstand transport and rough weather conditions at sea.

RESULTS AND DISCUSSION

Instrument Design

The previously mentioned characteristics of portability, low detection limits, rapid data acquisition, and multidimensional detection were all achieved by using an intensified 512 element photodiode array detector. When coupled to a flat field spectrograph this array can simultaneously detect the spatially dispersed emission spectrum over a 600nm window. The detection system used was Tracor Northern's (Middleton, WI) IDARSS system which consists of an intensified array, spectrograph, and multichannel analyzer.

Multidimensional detection is possible by the rapid acquisition of emission spectra at several different excitation wavelengths. A circular variable filter wheel was purchased from Optical Coating Laboratory (Santa Rosa, CA) to provide the excitation resolution. This filter wheel is an interference filter constructed such that the transmitted wavelengths vary linearly with the angular position of the wheel. The wavelength range is 400-700nm with the transmittance ranging from 20% at 400nm to 46% at 700nm. A 200 step stepping motor was obtained from Superior Electric (Bristol, CT) to drive the filter wheel.

Both the diode array and the stepping motor driven filter wheel were interfaced to an Apple II+ with 64 Kbytes

(8 bits/byte) of RAM, two floppy disk drives, and a CRT monitor. An Apple Super Serial® interface (Apple Computer, Cupertino, CA) provides the RS-232c serial communication link between the Apple II+ and the TN-1710 multichannel analyzer which controls the diode array. The stepping motor is accessed by a Cybernetic (San Gregorio, CA) CY512 stepping motor controller via a SSM (San Jose, CA) parallel interface. Software control of the stepping motor was provided through the CY512 by ASCII commands from the Apple II+.

Custom built, foam insulated cases with an ABS plastic outer shell protect the instrument in shipment and from environmental conditions during operation. These cases have been modified to accomodate electrical and water connections and to eliminate stray light. A diagram of the completed PMF is given (Figure 1).

Sensitivity

A comparative evaluation was made of the PMF, video fluorometer (VF) [3], and a Perkin-Elmer LS-5. The different modes of detection and signal readout used by the three instruments make a completely equal comparison difficult. However, Talmi [11] compared the detection sensitivity of a PMT, SIT vidicon, diode array, and intensified array by the calculation of signal-to-noise ratios, S/N, at peak maxima while approximately equating the total exposure times. Significant improvement of S/N for the arrays resulted from

longer integration times and detector cooling with cold water to reduce dark current noise. This method seemed to be the most satisfactory and informative comparison for our purposes and was, therefore, used in this study.

In order to obtain the S/N values, 100 replicate standard chlorophyll a (10^{-8} M) spectra were acquired. The mean value of the emission maxima was calculated as the signal. The noise at the peak was characterized by the standard deviation of the peak maxima. By calculating the S/N at the peak in this manner both photon flicker and dark current noise contributions were accounted for.

The experimental conditions and the S/N values obtained for the three fluorometers in our laboratory are given in Table 1. Exposure times were selected such that a fair comparison could be obtained under relatively normal conditions. As expected from Talmi's [11] results, the PMF with its intensified array is significantly more sensitive than either the LS-5 or VF. In addition, it must be pointed out that the exposure time for the LS-5 was for a 50nm scan while the PMF covered 600nm and the VF over 200nm in both excitation and emission dimensions. Therefore, both the PMF and VF are relatively more versatile than is apparent from Table 1 due to the multiplex advantage [12].

Detection Limit and Linearity

The calibration curve for standard chlorophyll a given

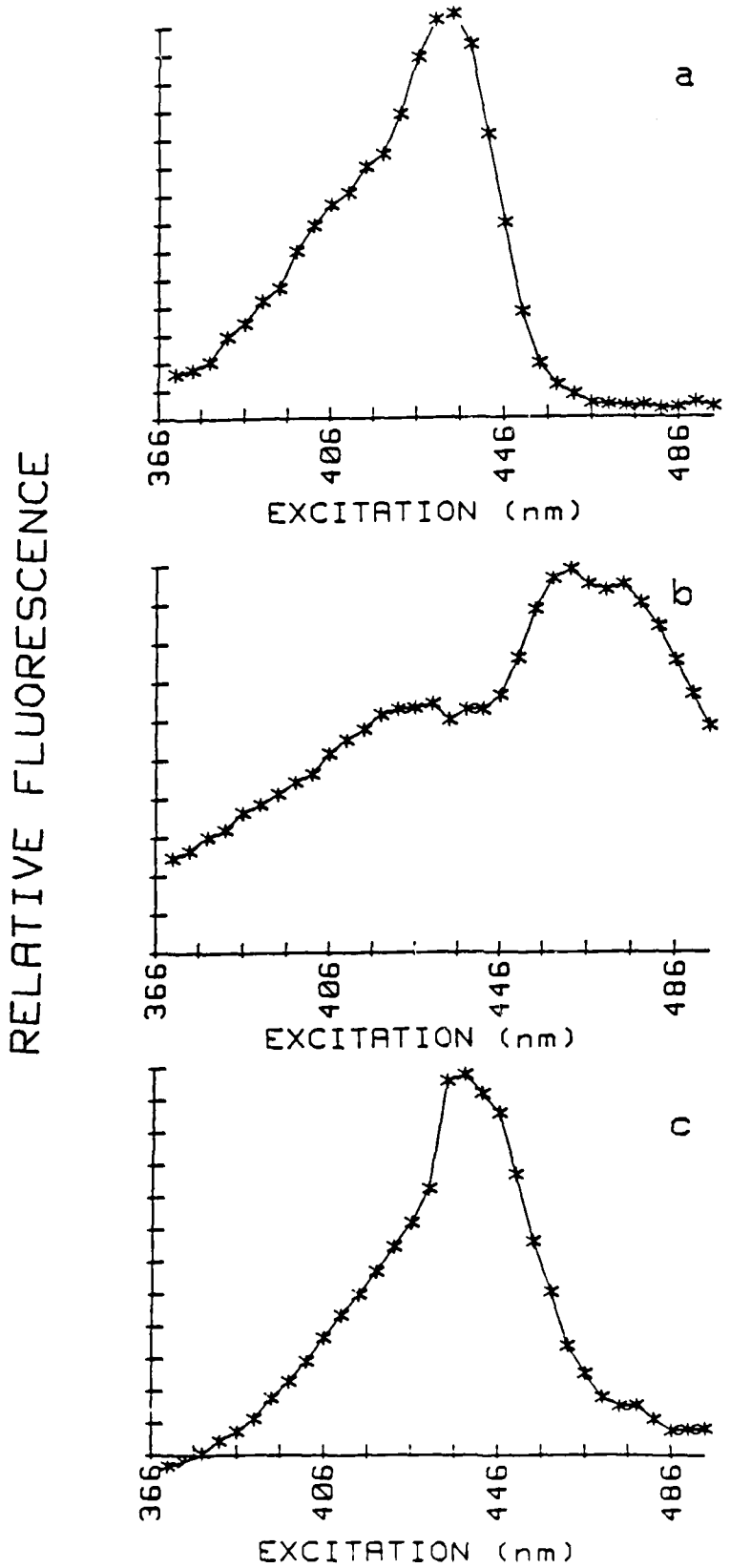
by the equation

$$y = 1.01 + 0.18x$$

shows linear response over three orders of magnitude in concentration with a correlation coefficient of 0.9994. The standard deviation of the slope (σ_m) was 0.198 and the standard deviation of the intercept (σ_i) was 126.8. The standard error of estimation, relative signal range, and number of points were 3.92, 234.1, and 7 respectively. These data were acquired with an exposure time of 0.205 sec. per scan. A detection limit of 5×10^{-12} M chlorophyll a was calculated for the S/N of 2 by accumulating 30 scans with an exposure of 1 sec. per scan. However, this detection limit is somewhat arbitrary due to the dependence of signal on integration time. By employing longer integration times a reduction in detection limit proportional to the square root of the integration time can be observed. Therefore, the limit of detection falls well below that necessary for studies of chlorophyl a in the ocean.

Computer Control and Data Acquisition

Control of the PMF is achieved using Applesoft BASIC ·ftware with some simple task-specific 6502 machine language s ·outines. Communication between the Apple II+ and the PMF ı ıccomplished through the interfaces described previously. A simple flowchart of a sample control program is given in Figure 2. Program modifications are easily performed to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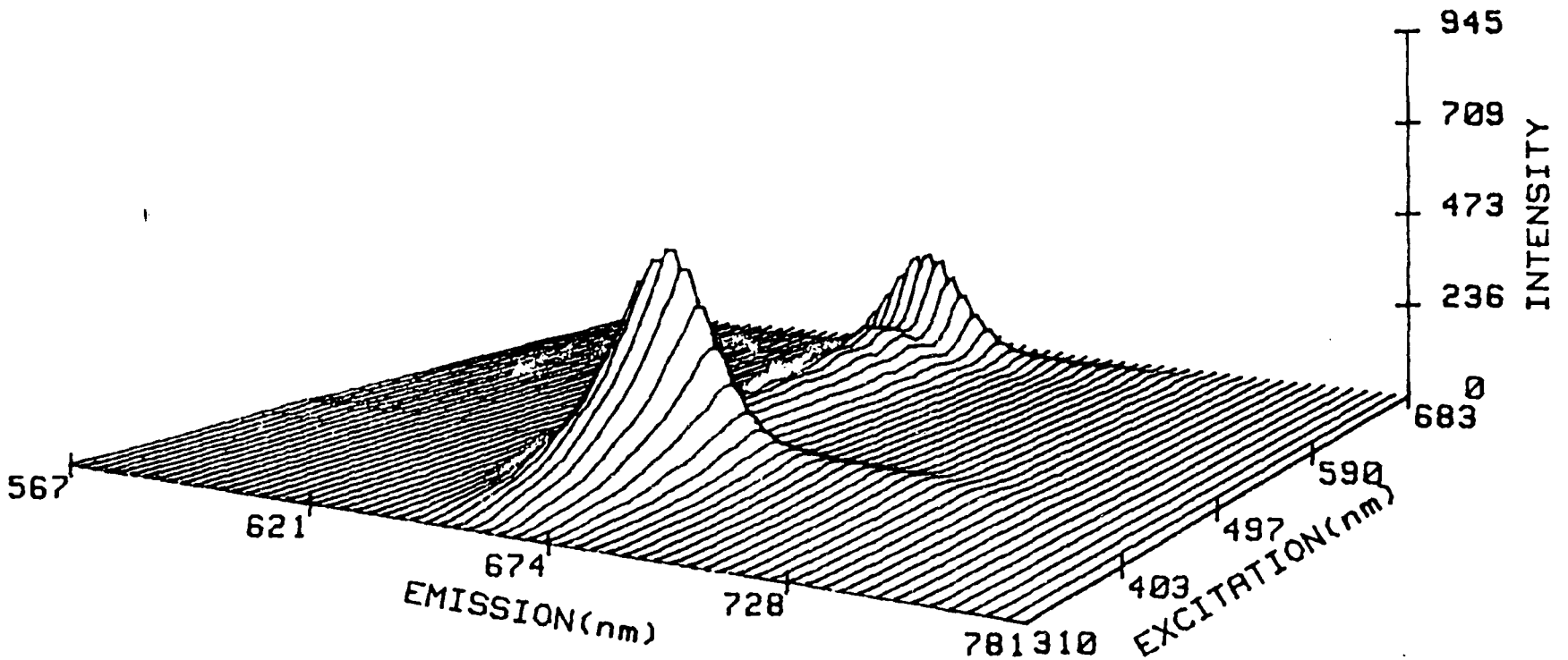
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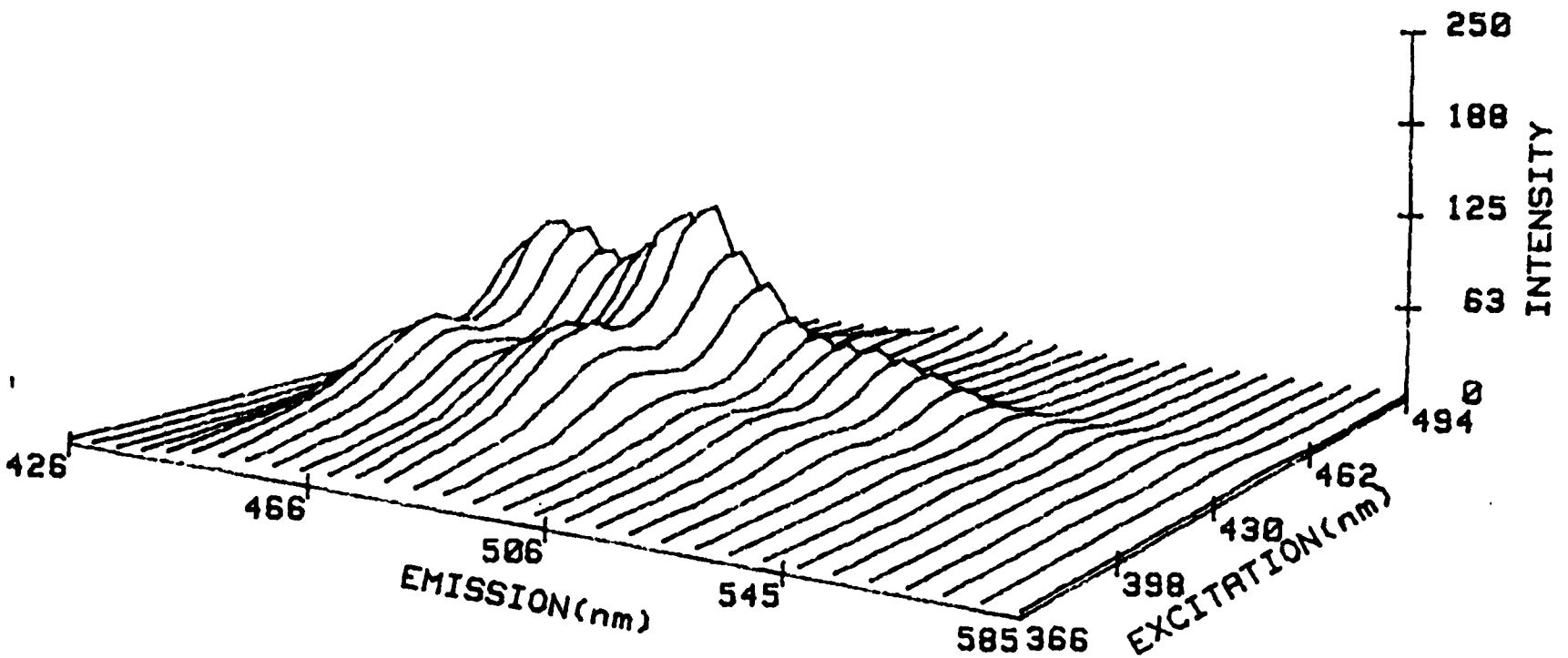
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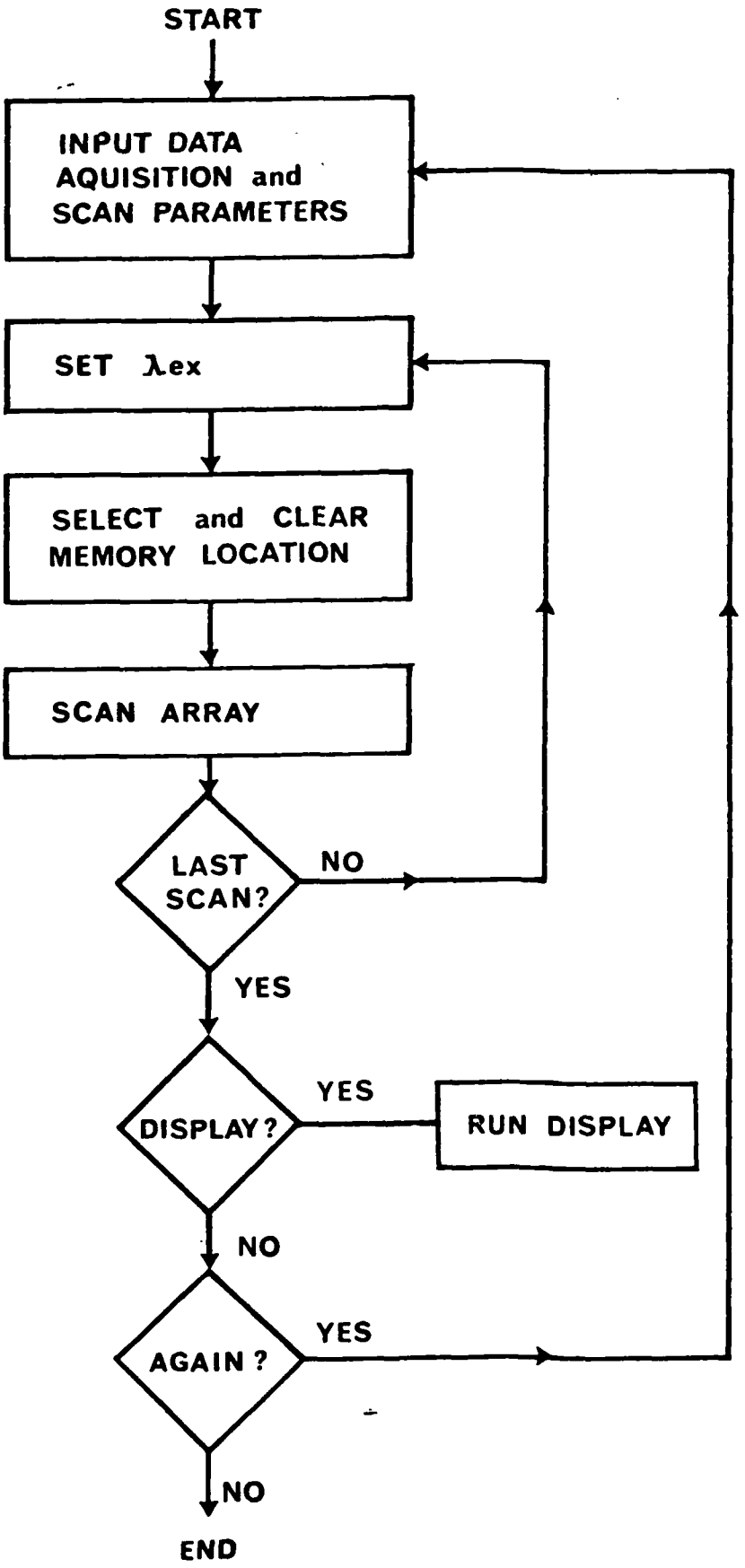
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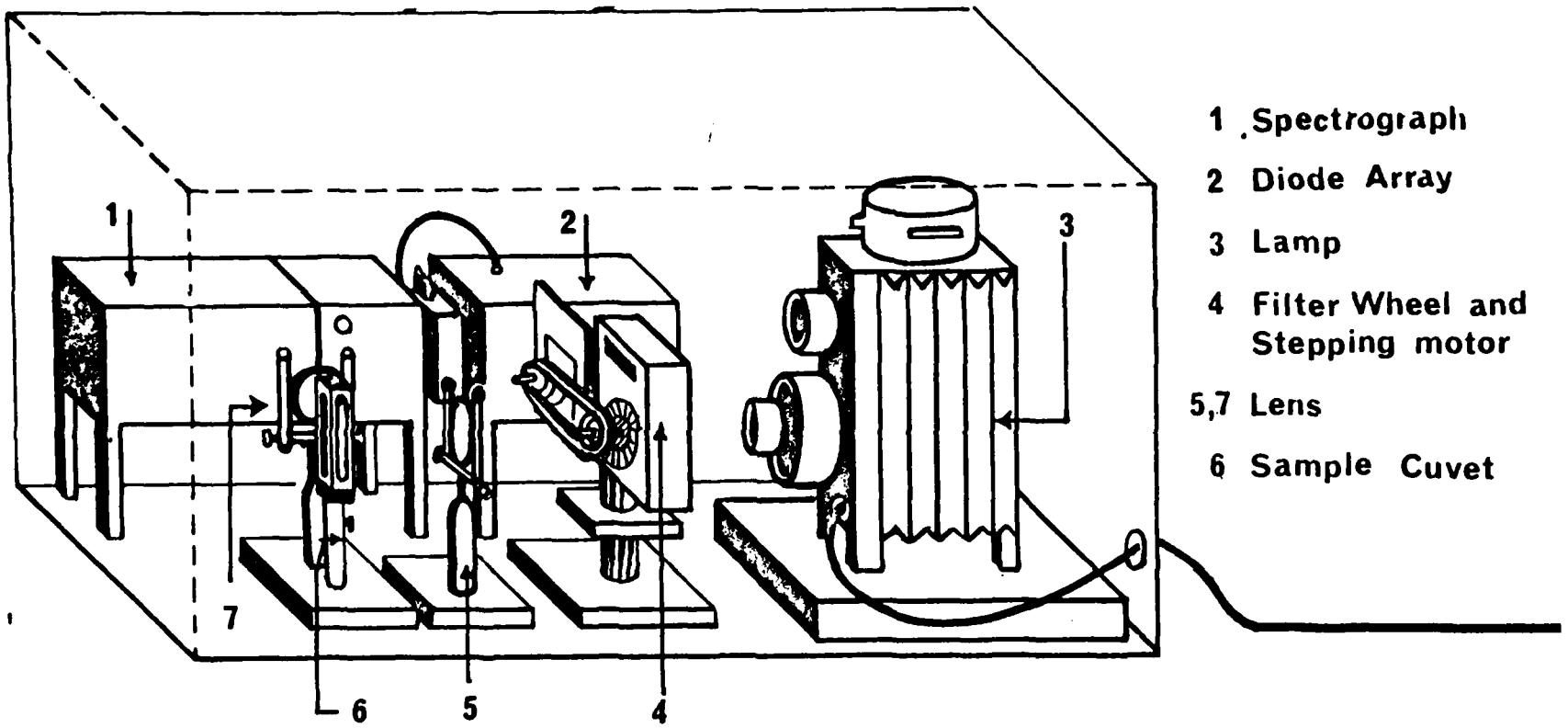

1 Spectrograph

2 Diode Array

3 Lamp

4 Filter Wheel and Stepping motor

5,7 Lens

6 Sample Cuvet

Table 2

PMF data acquisition and storage capabilities

# of emission spectra	16
Data acquisition time (min.)	1-4
Data storage time (min.)	3
Total time (min.)	4-7
Storage capacity (spectra/disk)	98

Table 1

Comparison of detection for chlorophyll a between the portable fluorometer, video fluorometer and Perkin-Elmer LS-5 *

	Integration Time	Average Signal at Peak (Counts)	Average Noise at Peak (Counts)	S/N At Peak
Portable Fluorometer	30 scans; 0.205 sec/scan ET = 6.15 sec	98,006.28	1120.84	87.44
LS-5	50nm/scan; 8nm/sec ET = 6.25 sec	88.24	1.21	72.93
Video Fluorometer	11 scans; 0.573 sec/scan ET = 6.31 sec	2,113.95	78.52	26.92

* Measurements were performed at peak; λex = 430nm, λem = 667nm

FIGURE CAPTIONS

Figure 1. Diagram of the portable multichannel fluorometer (PMF).

Figure 2. Sample BASIC program flowchart for data acquisition and control of PMF.

Figure 3. Two-dimensional fluorescence spectrum of perylene (10^{-8}M) acquired by the PMF and presented in an axonometric projection.

Figure 4. Axonometric projection of chlorophyll a (10^{-6}M) acquired by the VF.

Figure 5. Excitation spectra of a) standard chlorophyll *a* (λ_{em} = 670nm) b) in vivo Chlamydomonas reinhardi (λ_{em} = 685nm) c) extracted seawater sample (λ_{em} = 670nm).

Figure 6. Emission spectra of a) standard chlorophyll a (λ_{ex} = 430nm) b) in vivo Chlamydomonas reinhardi (λ_{ex} = 430nm) c) extracted seawater sample (λ_{ex} = 430nm).

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better sample characterization. By incorporating an intensified photodiode array, a scanning circular variable filter wheel, and a controlling microcomputer such spectral information can now be acquired. This fluorometer provides sensitive and rapid data acquisition and the capability of operating in a remote setting.

The PMF provides the mechanism whereby the characterization of phytoplankton populations can be easily explored. It enables the continuous acquisition of "total luminescence" spectra useful not only for "fingerprinting" populations but also for detecting the spectral distribution of fluorescent species in relation to topography.

CONCLUSION

The characteristics of portability, low detection limits, rapid data acquisition, multidimensional detection, automation capabilities, and rugged construction have been provided in the design of a portable, multichannel fluorometer (PMF) for less than $40,000. However, this cost has dropped approximately $10,000 in the past year due to advances in commercially available components. Conventional instruments are generally sensitive and possibly automated but lack the other attributes mentioned. It has been shown that multidimensional detection of fluorescence spectra is advantageous in multicomponent sample analysis. However, it is instrumentally limited by a lack of sensitivity and by restriction to laboratory investigations. The continuous determination of in vivo chlorophyll a as well as many other applications could benefit from a portable and sensitive fluorometer capable of rapidly acquiring multiwavelength spectra. Spectral information that is currently undetected could be useful in eliminating interferences and toward

which occurs at 430nm, is called the Soret band. It is the Soret band which is usually monitored during *in vivo* chlorophyll a determinations and so the following data will focus on this excitation band.

Characteristic spectra were acquired of the *in vivo* fluorescence of *Chlamydomonas reinhardi* which is a member of the *Chlorophyta* or "green algae" family. Spectra were also acquired of extracted chlorophyll a from real seawater samples. All of these spectra were obtained with the PMF for comparison with standard chlorophyll a spectra (Figures 5 and 6). Both of the extracted chlorophyll a spectra are similar to the standard spectra. However, the excitation band for the extracted sample is slightly broadened. This is likely due to the transfer of excitation energy from secondary pigments present in the phytoplankton to chlorophyll a which relaxes by fluorescence [13].

There is a significant difference between the excitation spectrum of the *in vivo* sample and the other two. A 15-20nm red shift of the emission profile is also characteristic of *in vivo* fluorescence [14].

These preliminary data confirm previously reported results [15] which indicate the excitation spectrum as a useful "fingerprint" of phytoplankton cultures. Further investigation is required to adequately determine the extent of spectral differentiation between phytoplankton cultures.

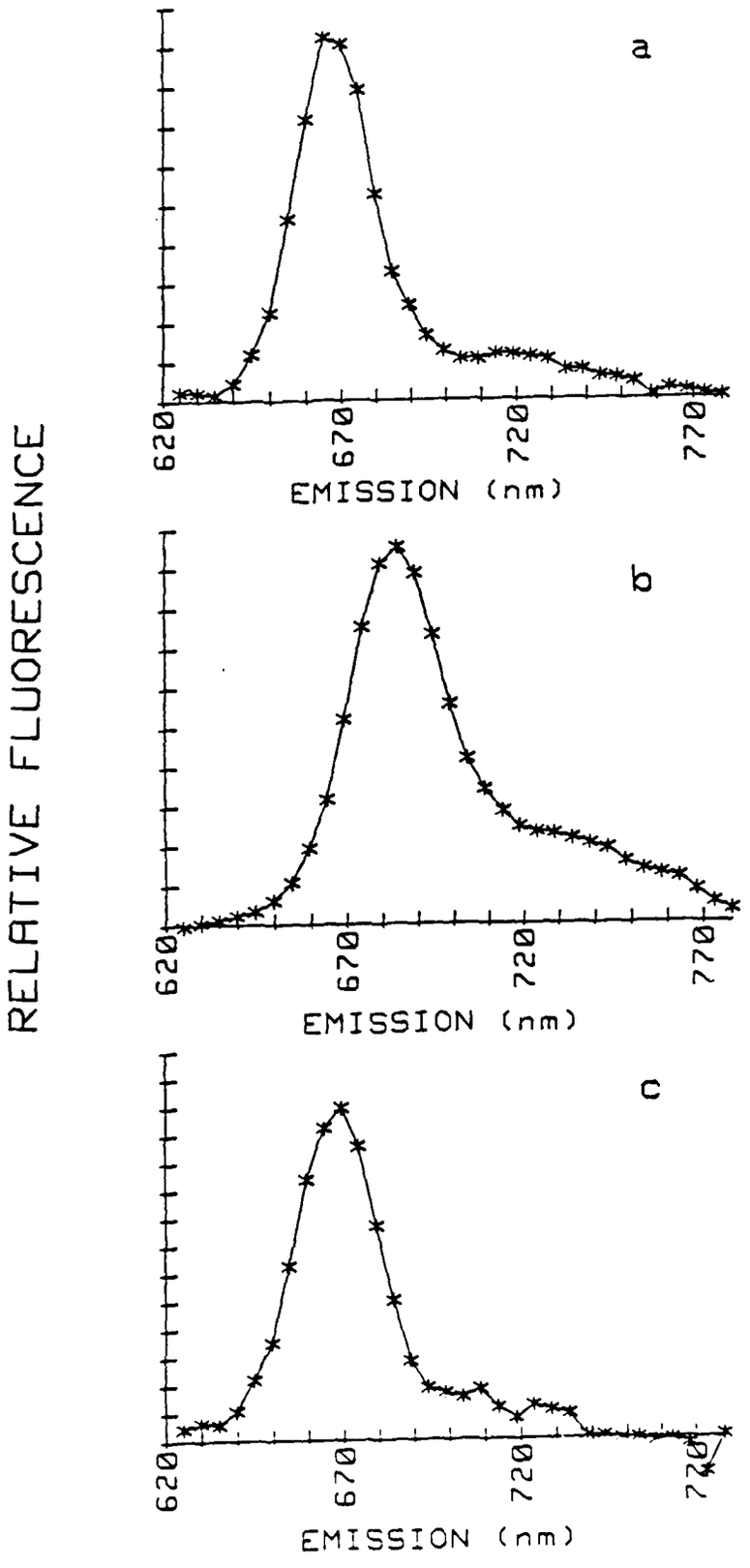
accomodate specific applications.

Data are acquired by sequentially gathering the emission spectrum at different excitation wavelengths as previously described. The TN-1710 multichannel analyzer contains 8 Kbytes (16 bits/byte) of RAM and therefore, can store up to 16 emission spectra at a time. After the data have been acquired by the TN-1710, they are transferred to the Apple II+ and stored on floppy disks as one-dimensional arrays. A list of the data acquisition and storage capabilities of the PMF are given in Table 2.

After the one-dimensional emission spectra have been stored, they can be recalled individually or accumulated into a two-dimensional matrix similar to that acquired by the VF. Figure 3 is such a two-dimensional spectrum acquired by the PMF. This capability provides the investigator with significant versatility. A complete two-dimensional spectrum can be rapidly generated when desired. However, for many applications only a few excitation wavelengths are needed, thus increasing the time resolution between spectra and reducing the data storage space required.

Preliminary Experiments

A total luminescence spectrum of standard chlorophyll a was acquired on the VF and displayed as an axonometric projection (Figure 4). There are five excitation bands and one major emission band. The most intense excitation band,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The multichannel fluorimeter described provides good sensitivity and rapid data acquisition. The advantages of multichannel fluorescence detection are discussed with special reference to the continuous monitoring of in vivo chlorophyll fluorescence in sea waters. Experiments on chlorophyll a determinations indicate a detection limit of 5×10^{-12} M with a linear range over at least three orders of magnitudes of concentration.

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